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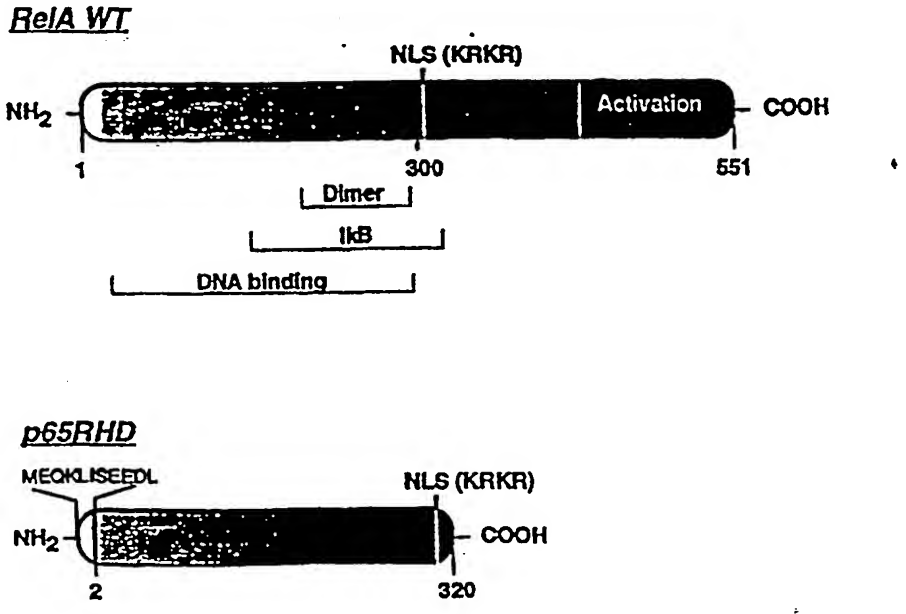

(57) Abstract

A method to render mammalian, particularly endothelial cells less susceptible to platelet- and leukocyte-mediated injury and inflammation is described, comprising genetically modifying the cells by inserting DNA encoding a transdominant inhibitor of a RelA (p65) protein, and expression functional inhibitor in the modified cells under cellular activating conditions, whereby RelA (i.e. NFκB) transactivation of genes is suppressed, and corresponding proteins and DNA therefor. The method, which can be carried out *in vivo*, *ex vivo* or *in vitro*, has use in allogeneic or xenogeneic transplantation as well as to treat systemic or local inflammatory conditions.

GENE THERAPY WITH MODIFIED p65 PROTEINS

Field of the invention

The invention relates to the fields of gene therapy and tissue and organ transplantation. It concerns genetic modification of endothelial cells, or other mammalian cells such as hematopoietic cells, to render them less susceptible to an inflammatory stimulus. In particular, it is addressed to genetic modification of endothelial or other mammalian cells to render them capable of expressing a protein which specifically inhibits NFκB, whereby NFκB-transactivation of inflammatory and other proteins is suppressed or inhibited under cell activating conditions.

It is also concerned with transplantation of genetically modified cells, or tissue or organs comprising such cells, capable of expressing the inhibiting protein; it most particularly is directed to methods of transplanting modified xenogeneic or allogeneic cells, tissue or organs; recombinant genes, proteins and vectors for accomplishing same; and the cells, tissue or organs, as well as non-human transgenic or somatic recombinant animals, so modified.

Background of the Invention

This invention relates to methods of suppressing mammalian (e.g. endothelial) cell activation, and in particular relates to allo- or xenotransplantation of endothelial cells, and tissues and organs containing them.

A major problem in the successful transplantation of organs between discordant species is hyperacute rejection of the organ, the main initiators of which are the antibodies and complement system of the organ recipient. In this respect, one approach to attaining prolonged graft life has been to prepare donor organs which express complement regulatory factors of the recipient.

However, the cells of the donor organ themselves, i.e. the endothelial cells, also give rise to rejection by stimulating coagulation in the recipient, and by undergoing physical and metabolic changes in a process known as "activation". In mammals, the endothelium (also known as the "vascular endothelium") consists of a layer of cells that line the cavities of the heart and of the blood and lymph vessels. Endothelial cells (EC) normally maintain vascular integrity and blood flow.

The process of activation of donor endothelial cells by graft recipient platelet- and leukocyte-mediated release of activating agents (e.g. cytokines such as IL-1α), has been described in the literature [Bach et al., Immunological Reviews 141 (1994) 5-30].

In particular, when "activated" by inflammatory stimuli such as a bacterial endotoxin comprising a lipopolysaccharide (LPS) or inflammatory cytokines (IL-1, TNFα), the EC tend to retract from one another, resulting in leakage of blood cells and plasma proteins (i.e. hemorrhage and edema), and loss of heparin sulfate and thrombomodulin, among other proteins, from the EC surface, in turn leading to coagulation and platelet aggregation. The next stage is characterized by induction in the EC of a number of genes and their products, including those coding for adhesion molecules that promote host leukocyte adhesion and extravasation, tissue factor that enhances the pro-coagulant phenotype of the surrounding cellular environment, cytokines and monokines that also contribute to the attraction and activation of leukocytes, interleukins, and other procoagulant, prothrombotic components of the coagulation system. Graft injury and loss seen in allograft and xenograft rejection, as well as graft preservation-induced endothelial damage, exemplify the vulnerability of endothelial cells, tissue and organs in the activated condition.

Considerable effort by workers in the art has been directed toward elucidation of agents which can control endothelial cell activation as well as activation of mammalian cells in general. However, there continues to exist a critical need for methods of preventing or minimizing the physiological processes associated with endothelial cell activation. In particular, there is a need to prolong graft organ survival, while minimizing toxicity and other adverse effects often seen with available activation- or inflammation-suppressing agents.

An identified transcription factor that induces transcription of a wide variety of genes associated with endothelial cell activation (e.g. immunomodulatory cytokines, cell surface receptors, procoagulant factors), by binding to specific decameric sequence motifs in enhancer and promoter elements of said genes (referred to as "κB" sequences), is "Nuclear Factor κB" (NFκB).

In its active form NFκB constitutes a dimer comprised of members of the Rel/NFκB family of proteins: RelA (p65), Rel (= c-Rel), RelB, $NF\kappa B_1$ (p50) and $NF\kappa B_2$ (p52). Almost all combinations of these subunits into homodimers and heterodimers have been isolated, and are reported to have different affinities toward κB sequence motifs. For example, p50 and p52 strongly bind such sequence motifs and, when dimerized, are found to contribute mainly to DNA binding rather than transactivation. On the other hand, p65 (RelA), Rel and RelB carry transcription activation domains of varying strengths, with less participation in binding. In particular, RelA (alternatively known in the art as "p65"), while having significantly weaker DNA binding activity than p50, has been found to be a potent transcriptional activator.

The Rel/NFκB proteins are characterized in having a highly homologous sequence of approximately 300 amino acids referred to as the "Rel Homology Domain" (RHD). This homology also occurs across species; for example, murine and human RelA (p65) have been found to be strongly homologous. Biochemical analysis has shown that the predominant form of NFκB in mammals (e.g. humans) is a heterodimer comprising p50 and p65, subunits. The NFκB heterodimer is constitutively expressed in the cytoplasm of cells. In unstimulated cells, NFκB is sequestered in an inactive form in the cytoplasm by binding to an inhibitor, namely, IκBα, via the RelA (p65) subunit. In this trimeric form, the IκBα appears to mask the nuclear localization signal (NLS) within the Rel homology domain of the p65 and p50 subunits of NFκB. However, upon stimulation of cells with specific agents such as IL-1, TNFα, or lipopolysaccharide (LPS), the IκBα protein is rapidly phosphorylated and proteolytically degraded, liberating the NFκB dimer and thereby unmasking the NLS, and facilitating the rapid translocation of NFκB to the nucleus. In essence, NFκB functions as a transducer of cytoplasmic signals to the nucleus by a translocation mechanism. Once in the nucleus, NFκB binds to available κB sites in control

elements of the nuclear DNA, and induces transcription of the underlying gene. Inasmuch as many of the genes associated with endothelial cell activation, such as inflammatory genes, contain at least one κB site, the activation of NFκB ultimately leads to transactivation of genes involved in the inflammatory process. By "transactivation" is meant the regulation of gene activation and transcription by a cellular factor (e.g. NFκB) acting in trans (i.e. without being covalently bound to the gene), by binding to or otherwise influencing the control elements (e.g. promoters, enhancers) which regulate the gene in cis (i.e. by covalent bonding to the coding part of the gene) [see e.g. Baeuerle and Henkel, Ann. Rev. Immunol. 12 (1994) 141-179; Fujita et al., Genes & Dev. 6 (1992) 775-787; Urban et al., EMBO J. 10 (1991) 1817-1825].

One approach to effect inhibition of induction of inflammatory genes under activating conditions has been to stabilize the NFκB-IκBα trimer by protecting IκBα against phosphorylation and proteases, and thus prevent migration of NFκB to the nucleus. However, anti-oxidants as well as proteasome inhibitors suffer from non-specificity. At present there is a lack of available inhibitors of NFκB that are both specific and effective. It has remained an objective in the art to identify means of specifically preventing or suppressing induction of inflammatory genes, especially under endothelial cell activating conditions.

Summary of the invention

It has now been found that certain modified proteins can function as potent inhibitors of NFκB under cellular activating conditions. Such proteins in general have the properties of:

(a) binding to κB sequences in control elements (e.g. promoters, enhancers) of cellular DNA;

(b) dimerizing with another protein containing a Rel homology domain; and

(c) being substantially incapable of transactivating a gene normally subject to transactivation by NFκB.

Such proteins are typically heterologous (i.e. not native) to the cell, and may be under the control of one or more promoters and/or enhancers which are also heterologous to the cell.

In particular, it has now been found that the RelA (p65) protein, when modified to disable its transactivation domain, can function as a potent inhibitor of NFκB -induced transcription of inflammatory genes, and that such modified RelA (p65) protein can competitively bind to DNA sequences bearing a κB motif; as against the predominant form of NFκB comprising a heterodimer of RelA (p65) and p50. Specifically, it has been observed that reporter gene constructs known to be transcriptionally activated by endogenous RelA (p65) are rendered resistant to such activation by co-expression of the above modified RelA (p65) protein.

Suppression of activation by the above modified protein is highly specific. For example, transcriptional induction of the HIV-LTR by HTLV-1 Tax (which activates NFκB) is found to be fully inhibited, while NFκB-independent HIV Tat-induced transcription from the HIV-LTR is not so inhibited. Transcriptional inhibition by the altered protein of the invention is unexpectedly potent, even at low concentrations of the protein, leading to effective suppression of induction of cytokine-inducible genes such as tissue factor, E-selectin, IL-8, IL-6 and IκBα, many of which are associated with inflammation. In studies in which the endogenous, wild-type RelA (p65) and the altered protein of the invention are provided on an essentially equimolar basis to cultured endothelial cells, inhibition has been found in many instances to be 50% or greater, even 75% or greater, 90% or greater, and even 95% or greater, or 100%.

As such, the modified RelA (p65) protein of the invention functions as a "dominant negative derivative," or alternatively, a "transdominant inhibitor" of the endogenous gene. The terms "dominant negative" or "transdominant inhibitor" are intended to refer to the capability of suppressing a normal function of an endogenous protein.

The invention concerns modified human p65 proteins transdominantly inhibiting the wild-type p65 protein, preferably having the transcription activation domain of the wild-type protein substantially dysfunctional or deleted, and corresponding DNA coding therefor, optionally with ancillary sequences for e.g. quantification or recognition, such as a portion

of human c-myc, and also concerns the use of such modified proteins and DNA in, i.a., the preparation of a corresponding medicament.

Specifically, the invention concerns proteins, and corresponding DNA coding therefor, comprising or coding for, essentially, amino acids 1-320 or 2-320 of the wild-type human p65 amino acid sequence, optionally with ancillary sequences for e.g. quantification or recognition, such as a portion of human c-myc. They have e.g. the amino acid and nucleotide sequence disclosed in Seq. Id. No. 1 and No. 2, or a sequence obtained by e.g. adding or replacing one to several amino acid residues in Seq. Id. No. 2.

The invention also concerns vector constructs for achieving delivery of modified proteins as defined above, or of corresponding DNA coding therefor, to appropriate recipient cells, tissue or organs, such as vectors for delivering cDNA encoding a modified RelA (p65) under the control of a regulable (e.g. inducible) promoter into an endothelial or other mammalian cell; they are preferably comprising a regulable element such as a tetracycline-inducible promoter.

The gene (protein) of the invention may be prepared by well-known recombinant techniques, e.g. by effecting additions, substitutions or deletions in the nucleotide (amino acid) sequence of the transactivation domain of the RelA gene (protein), so as to render the transactivation domain substantially dysfunctional. By "substantially dysfunctional" is meant that transactivation of at least one inflammatory protein (e.g. E-selectin) is reduced by at least 50%, and preferably by at least 75%, and more preferably by at least 90%, and even 100%, relative to transactivation by an equimolar amount of the wild-type protein.

Preferably, the modified gene (protein) of the invention is a deletion mutant (i.e. truncation) of the naturally occurring gene (protein), whereby at least a portion of the transactivation domain of the wild-type gene (protein) has been excised. Preferably, the altered gene (protein) of the invention consists essentially of the Rel homology domain of the naturally occurring RelA (p65) protein.

While certain mutants derived from RelA (p65) have been prepared by workers in the art [see Beg et al., Genes & Dev. 6 (1992) 1899-1913; Sun et al., Science 259 (1993) 1912-1915], there has been no apparent demonstration of utilization of mutants as a transdominant negative competitor of NFκB in activated endothelial cells.

It has also been found that transcription and expression of the truncated RelA-derivative can be achieved in a regulable manner, in particular, using a tetracycline-dependent expression system. For example, tetracycline-induced expression of p65RHD in stably transfected endothelial cells has been found to inhibit LPS-mediated induction of endogenous genes such as E-selectin, P-selectin and IκBα.

Therefore, in one aspect, the invention relies on gene therapy techniques, utilizing in its more particular aspects a recombinant gene encoding a mutant RelA (p65) derivative, to suppress or block NFκB-induced activation of mammalian (e.g. endothelial) cells susceptible to an inflammatory or immunological stimulus.

Inhibition of NFκB can reduce smooth muscle cell proliferation in addition to inhibiting endothelial cell activation [Autieri et al., Biochem. & Biophys. Res. Comm. 213 (1995) 827-836], thereby yielding reduced atherosclerosis and increased graft survival.

Accordingly, the invention in its broader aspects concerns a method of genetically modifying mammalian (e.g. endothelial) cells to render them less susceptible to an inflammatory or immunological stimulus by conferring on the cells the capability of expressing a transdominant inhibitor of endogenous NFκB, whereby NFκB-transactivation of genes is suppressed under activating conditions, as well as the use of such modified cells in the preparation of a medicament for suppressing NFκB-transactivation of genes under activating conditions.

The invention also comprises a method of controlling cellular (e.g. endothelial cell) activation in a mammalian patient, comprising genetically modifying cells of the patient by inserting therein DNA encoding a transdominant inhibitor of endogenous NFκB, and expressing functional inhibitor in the nucleus of the cell, whereby NFκB-transactivation of genes is suppressed, as well as the use of such modified cells in the preparation of a medicament for controlling cellular (e.g. endothelial cell) activation in a mammalian patient. By "endothelial cell activation" or mammalian cellular activation is meant transcriptional upregulation and synthesis of inflammatory proteins, adhesion molecules and coagulants (also known as "type II activation"). A generally accepted indicator of type II EC activation is an elevated level of E-selectin transcription. Preferably the cells or tissue are

modified in vivo, i.e. by insertion of a vector comprising the cDNA in the cells while they remain in the body of the patient. Alternatively, the cells or tissue may be extracted from the subject, genetically modified ex vivo by insertion of DNA, and then grafted into the subject. The subject is a vertebrate, in particular a mammal, such as of the porcine or bovine species, but may also be a primate, and in particular, a human.

It will be apparent that such a therapy will be useful to alleviate inflammatory conditions in a patient (i.e., by syngeneic therapy), and also to moderate complications occurring in connection with organ transplantation, especially where the graft recipient is human.

Thus in a further aspect, the invention comprises a method of transplanting donor allogeneic or xenogeneic mammalian (e.g. endothelial) cells, or tissue or organs to a mammalian recipient in whom such cells, tissue or organs are subject to inflammatory or immune activation, which comprises:

(a) genetically modifying the donor cells or progenitor cells thereof or tissue or organ by inserting therein DNA encoding a transdominant inhibitor of the RelA (p65) protein;

(b) implanting the resultant modified donor cells, tissue or organs into the recipient; and

(c) expressing in the resultant modified cells, tissue or organ, functionally active transdominant inhibitor, whereby transcriptional activation of inflammatory genes therein is suppressed.

Activation occurs as a result of contacting of the host blood or plasma with the donor cells, tissue or organs.

Steps (a) and (b) may be carried out in either order; that is, the donor allogeneic or xenogeneic cells, tissue or organs may be modified or genetically engineered (e.g. by transfection, transduction, transformation or the like) prior to, or alternatively after, implantation into the recipient.

For example, endothelial cells of a pig may be genetically modified by insertion of DNA encoding a heterologous protein comprising a transdominant inhibitor of the RelA protein of said cell, under the control of a promoter. The modified cells or tissue or organ

may then be grafted into a human recipient. Once transplanted, the donor porcine cells or tissue or organs express functional heterologous protein, preferably on a regulable basis. The heterologous protein inserted into such pig cells may be a mutant of a mammalian Rel/NFκB protein, e.g. RelA (p65); given the homology between proteins of the Rel/NFκB family, the mutant human protein is able to exert transdominant inhibition of the corresponding wild-type porcine protein in the porcine cell.

In one embodiment, a heterologous gene, e.g. a modified human RelA'(p65) gene in a suitable vector will be used to modify porcine donor cells or organs in vivo, to render them transgenic or somatic recombinants, for transplantation purposes.

Somatic recombinant or transgenic donor animals can be obtained by well-known techniques. The somatic cells of the animal can be appropriately modified in vivo to provide a somatic recombinant. Alternatively, fertilized oocytes of non-human mammals can also be modified by well-known procedures so as to produce a true transgenic expressing in its cells the desired protein. Cells, tissues and organs which can express the desired functional protein once transplanted into a recipient (e.g. human) can then be recruited from the donor animal for transplantation .

Donor cells or tissue can also be genetically modified ex vivo, whereby cells, tissues or organs extracted from the donor (e.g. pig) and maintained in culture are genetically modified as described above, and then transplanted to a recipient (e.g. a human), where the graft can then express the desired functional protein. It is preferred that the genetic modification be done in vivo.

A regulable expression system is disclosed hereunder, particularly for use in preparing transgenic animals expressing the protein of the invention. By "regulable" is meant that protein expression, whether increased or decreased, is dependent on the presence, or addition of, a given substance. An embodiment of "regulable" expression comprises "inducible" expression, i.e. whereby gene expression is increased by addition of a stimulus.

According to a further aspect of the invention, there are provided graftable mammalian (e.g. endothelial) cells, tissue or organs comprising DNA encoding a transdominant inhibitor of the endogenous RelA (p65) protein, from a donor species, the

cells, tissue or organ being modified to regulably or constitutively express a transdominant negative inhibitor of a cellular RelA (p65) protein when transplanted into a graft recipient of the same or different species as the donor.

The invention also includes mammalian (e.g. human) cells transformed by a vector comprised a modified RelA (p65) gene which is capable of transdominantly inhibiting the wild-type RelA (p65) protein. Examples of such cells include hematopoietic cells such as lymphocytes or stem cells.

In its additional aspects, the invention provides a non-human transgenic mammal comprising DNA encoding a transdominant inhibitor of a RelA (p65) protein, namely having cells (e.g. endothelial) or tissue or organs comprising these cells, accordingly modified; and a method of preparing such non-human transgenic mammal.

Description of Sequence Identifiers

Seq. Id. No. 1: Nucleotide sequence of the vector construct prepared in Example 1, comprising the deletion mutant p65RHD at nucleotides 40-996 (i.e. corresponding to positions 83-1039 of the sequence disclosed by Rubin et al., infra; GenBank accession number M62399) with the codon for Met at position 1 and the c-myc sequence at nucleotides 4-33 (nucleotides 34-39 being derived from the cloning site.)

Seq. Id. No. 2: Amino acid sequence encoded by the vector construct prepared in Example 1, comprising the deletion mutant p65RHD at residues 14-332 (corresponding to residues 2-320 of the sequence disclosed by Rubin et al., infra) with Met at position 1 and the c-myc sequence at residues 2-11 (residues 12 and 13 being derived from the cloning site).

Explanation of the Figures

Figure 1: Schematic drawing of human RelA (p65) wildtype ("RelA WT") and p65 RHD ("p65RHD") constructs ["NH_2" and "COOH" refer to the amino and carboxy terminus, respectively; "RHD" refers to the Rel homology domain; "NLS (KRKR)" refers to the nuclear localization signal (bracketed)]. The brackets below the RelA WT diagram indicate the extent of the dimerization domain ("Dimer"), the IκBα interaction domain ("IκB") and the DNA binding domain ("DNA binding"). The numbers refer to the amino acid residues of wild-type human p65, starting with 1 at the amino terminus.

Figure 2: Repression by the p65 RHD construct of RelA ("p65 WT")-mediated activation of ECI-6 reporter in BAEC. Different amounts of pRC.CMV/p65RHD ("p65 RHD") were cotransfected with pCMV4TΔp65 (p65 WT).

Figure 3: Repression by p65 RHD construct of LPS-induced activation of Tissue Factor (A), ECI-6 (B) and ELAM-1 (C) reporter in BAEC. Different amounts of pRC.CMV/p65 RHD (p65 RHD) were cotransfected.

Figure 4: Tetracycline-regulated expression of p65RHD from pUHD10-3/RHD. Repression by p65 RHD construct of RelA-mediated activation of ECI-6 reporter in BAEC in the absence of tetracycline.

Figure 5: Doxycycline-regulated expression of p65RHD in stably transfected BAEC. BAEC were transfected with pUHD172-1neo and pUHD10-3/RHD and selected on G418. Expression of p65RHD was induced with 2 µg/ml doxycycline and whole cell extracts were prepared at time 0, 8, 16, 24, 48 and 72 hours, separated by SDS-PAGE, blotted on a PVDF membrane and probed with a polyclonal antibody directed against a N-terminal epitope of RelA. Bands were revealed using a HRP-conjugated anti-rabbit-IgG antibody and an enhanced chemiluminescent system. Repression of endogenous E-selectin, P-selectin and IκBα induction by TNF:
Top: Northern blot analysis for the indicated genes. Bottom: Western blot analysis decorated with anti-p65 (N-terminus-specific) antibody.

Definitions

"Graft," "transplant" or "implant" are used interchangeably to refer to biological material derived from a donor for transplantation into a recipient, and to the act of placing such biological material in the recipient.

"Host or "recipient" refers to the body of the patient in whom donor biological material is grafted.

"Allogeneic" refers to the donor and recipient being of the same species (as well as "allograft"). As a subset thereof, "syngeneic" refers to the condition wherein donor and recipient are genetically identical. "Autologous" refers to donor and recipient being the same individual. "Xenogeneic" (and "xenograft") refer to the donor and recipient being of different species.

Human RelA (p65) protein (gene) refers to the protein (gene) having the amino acid (cDNA) sequence disclosed by Ruben et al., Science 251 (1991) 1490-1493.

Detailed Description

NFκB/Rel proteins share a highly homologous sequence of approximately 300 amino acids referred to as the "Rel homology domain" (RHD). The RHD contains sequences necessary for DNA binding, nuclear localization, dimerization, and IκB binding, but lacks the transcriptional activation function that is found in domains C-terminal to the RHD in RelA, Rel, and RelB.

A cDNA sequence and deduced amino acid sequence of human RelA(p65) obtained from Jurkat T cells was identified by Ruben et al., supra. The amino acid sequence of 551 residues (including the termination codon and the putative initiation codon) encodes a protein of 60.2 kd. The Rel homology domain has been localized to amino acids 1-300, or alternatively 1-320 (if the complete IκB recognition site is included).

The modified recombinant protein of the invention preferably comprises a trans-acting dominant negative derivative of a Rel protein. It comprises at least a portion of the Rel homology domain (RHD), such that it is capable of a) binding to a gene regulatory region having affinity for one or more members of the Rel family of proteins; and b) forming a homo- or hetero-dimer with a second Rel protein. It also has a substantially dysfunctional or deleted transcriptional activation domain, such that it is substantially incapable of inducing transcription of genes (e.g. P-selectin, tissue factor or ELAM-1) which are normally inducible by one or more of the Rel family proteins. It can be derived recombinantly by rendering the endogenous protein substantially unable to transactivate a gene under the control of a κB-containing control domain. Preferably, it is a C-terminal deletion mutant of the p65 protein, whereby at least a portion of the transcriptional activation domain of the naturally occurring p65 protein has been excised. Alternatively, the protein (gene) may comprise one or more of the amino acids (nucleotides) normally constituting the transactivation domain of the wild-type protein (gene), so long as the transactivation function is rendered dysfunctional, e.g. by one or more mutations or substitutions of amino acids (nucleotides) or by deletions which are other than truncations. The resultant mutant protein functions to suppress or block induction of genes whose expression is dependent on the transcription factor, NFκB.

In a preferred embodiment, the protein, and the DNA encoding it, comprise the Rel homology domain of the p65 (i.e. RelA) protein, but is essentially free of a transcription activation domain. The transcriptional activation domain of p65 comprises at least two distinct segments in the C-terminal third. The first consists of a short but strongly transactivating sequence in the very C-terminus, characterized by a putative α-helix in the last 20-24 amino acids. An additional transactivating sequence encompasses amino acids between position 441 and 518 of the naturally occurring protein [Schmitz and Baeuerle, EMBO J. 10 (1991) 3805-3817]. It has further been indicated that amino acid residues between 415 and 550 (containing a leucine zipper-like motif in residues 435-459) constitute a transactivation domain.

Any or all of the foregoing amino acid sequences may be excised from the endogenous protein sequence to render a deletion mutant having impaired transactivation function. In one embodiment, such a mutant protein lacks the carboxyl 250 amino acids of native human RelA (p65). In other embodiments, the mutant derivative protein may comprise residues 1-400, or 1-350, or 1-320 of the native human RelA protein.

Alternately, the amino acid sequence of the modified RelA (p65) protein is substantially dysfunctional with respect to transactivation, and is at least 70%, preferably at least 80%, and more preferably at least 90% (and even more preferably at least 95%) homologous to the Rel homology domain of the native p65 protein. Amino acid residues 222-231 contribute to the formation of homodimers and heterodimers with p50 [Ruben et al., Molecular and Cellular Biology 12 (1992) 444-454], and are therefore preferably conserved in the altered protein. In general, a transdominantly acting protein of the invention can be prepared by truncating from the wild-type p65 sequence the region which is carboxy-terminal to the NLS.

Preferably, the modified protein consists essentially of the Rel homology domain, e.g. consists essentially of residues 1-320 or 2-320 (where Met is at position 1) of the native human protein, either of which is referred to herein as "p65RHD".

The κB motifs to which the RelA (p65) binding domain specifically binds, vary somewhat from gene to gene. An example of a generic κB DNA sequence comprises the following: 5'-GGGPuNNPyPyCC-3', where Pu is a purine nucleotide (i.e. adenine or guanine); Py is a pyrimidine base (i.e. thymine or cytosine); and N is any of adenine, cytosine, thymine or guanine.

According to the invention, a mammalian (preferably an endothelial) cell is transformed by a vector comprising the modified RelA (p65) gene. The gene is taken up and becomes resident in the nucleus of the cell, where the protein is expressed.

Once expressed, the protein is likely to form a homodimer with another altered RelA (p65) protein through interaction of the respective Rel homology domains. It is believed that under cellular activating conditions, when NFκB is freed from its association with IκBα, and translocates to the nucleus, the altered RelA (p65) protein, or homodimer thereof, has saturated the κB sites to render them incapable of effectively binding the NFκB heterodimer necessary for transactivation. The competitive binding activity, unexpectedly, suppresses to a high degree the transactivation function of NFκB even at low relative concentration levels of the altered protein.

Therefore the present invention broadly contemplates a method of rendering endothelial cells or other mammalian cells, or tissue or organs less susceptible to activation or dysfunction in response to an immune or inflammatory challenge by modifying the cells or tissue or organs by inserting therein DNA encoding a transdominant inhibitor of NFκB in operative association with a suitable promoter, and expressing functional transdominant inhibitor protein by the modified cells at effective levels under normal cell activating conditions.

The above modification of cells includes introduction of heterologous protein (or DNA) having the indicated activity. The protein encoding sequence is operably linked with a promoter sequence, which is typically also heterologous to the cell. The promoter may be constitutive or regulable (e.g. operate in an appropriate inducible manner).

In one embodiment the modified cells of the invention express the protein constitutively, i.e. continuously. Thus the DNA coding sequence is operably linked to a promoter sequence expressing the protein constitutively in said cell.

Alternatively, the modified cells express the protein on a regulable (e.g. inducible) basis, i.e. the protein coding sequence is operably linked to an inducible promoter, such that the protein can be expressed immediately before or following cell activation, or on demand in response to a predetermined external stimulus.

Examples of tetracyline-regulable systems particularly suitable for preparation of transgenic animals, have been disclosed by Furth et al., PNAS 91 (1994) 9302-9306 (tetracycline-repressible) and Gossen et al., Science 268 (1995) 1766-1769 (tetracycline-inducible) (see also Gossen and Bujard, USP 5'464'758) and have been found effective to regulate expression of the transdominant-acting protein of the invention. In particular, an inducible tetracycline system comprises:

(a) a first expression plasmid which expresses constitutively (either ubiquitously or tissue-specifically) a fusion protein between the bacterial tetracycline repressor (which in its native form is inhibited by Tet and in its mutant form is dependent on Tet), and a eukaryotic transcription activation domain (e.g. VP16), called tTA; and

(b) a second expression plasmid which contains multiple binding sites (TetO) for the bacterial tetracycline repressor followed by a minimal promoter (inactive by itself) and the gene to be expressed in a regulated manner (e.g. p65RHD).

Depending on which form of the tetracycline repressor (repressible or inducible) is used, expression of the gene of interest can either be turned off or turned on by tetracycline. Preferably, expression of the heterologous p65RHD gene from the cells of a subject (e.g., a transgenic mouse) is rendered inducible by administration of tetracycline, or an analog such as doxycycline, to the subject.

Thus the invention comprises a method for inhibiting the dysfunctional or activation response of vascular endothelial cells, or tissue or organs to an inflammatory or immune stimulus in a patient in need of such therapy, comprising modification of these cells, or tissue or organs in the patient as described above.

In a further embodiment, the invention comprises a method for inhibiting graft transplant rejection in a patient, which comprises:

(a) modifying donor mammalian (e.g. endothelial) cells, or tissue or organs comprising these cells, in vivo or in vitro by introducing therein DNA encoding p65RHD under the control of a suitable promoter;

(b) grafting these donor cells, tissue or organs into the patient; and

(c) expressing in these donor cells, tissue or organs, in the presence of cellular activating factors, functional p65 RHD protein.

The donor species may be any suitable species which is the same or different from the recipient species and which is able to provide the appropriate endothelial cells, tissue or organ for transplantation or grafting. In one embodiment, recombinant human protein is expressed from cells of a non-human mammalian species, which cells have been placed or grafted into a human recipient. For human recipients, it is envisaged that pig donors will be suitable, but any other mammalian species (e.g. bovine or primate) may also be suitable.

For example, porcine aortic endothelial cells (PAEC), or the progenitor cells thereof, can be obtained from porcine subjects, genetically modified, and either re-implanted into the autologous donor or an allogeneic recipient, or grafted into another subject of a different species (e.g. human).

The donor cells, tissue or organs may be transgenic or somatic recombinants in the sense that they contain and express DNA encoding a modified RelA protein. The modified RelA protein may be a derivative of the wild-type protein which is endogenous to the donor cell or donor species, or may be a derivative of a wild-type protein which is native to the species of a graft recipient in whom they are implanted, as is readily ascertainable by one of skill in the art. Such cells, tissue or organ may continue to express the desired protein indefinitely for the life of the cell. Modification of mammalian, e.g. endothelial cells according to the invention can be by any of various means known to the art. In vivo direct injection of cells or tissue with DNA can be carried out, for example. Appropriate methods of inserting foreign cells or DNA into animal tissue include microinjection, embryonic stem (ES) cell manipulation, electroporation, cell gun, transfection-k, transduction, retroviral infection, etc. Genes can be inserted into germ cells (e.g. fertilized ova) to produce transgenic non-human animals bearing the gene, which is then passed on to offspring. Genes can also be inserted into somatic/body cells to provide somatic recombinants, from whom the gene is not passed on to offspring.

In one embodiment, gene transcription is subject to an inducible promoter, so that expression of the recombinant protein can be delayed for a suitable period of time prior to grafting. In another embodiment, the gene is inserted into a particular locus, e.g. the thrombomodulin or P-selectin locus. Subsequently, the construct is introduced into embryonic stem (ES) cells, and the resulting progeny express the construct in their vascular endothelium.

For gene delivery, retroviral vectors, and in particular replication-defective retroviral vectors lacking one or more of the gag, pol, and env sequences required for retroviral replication, are well-known to the art and may be used to transform endothelial cells. PA 317 or other producer cell lines producing helper-free viral vectors are described in the literature. A representative retroviral construction comprises at least one viral long terminal repeat and promoter sequences upstream of the nucleotide sequence of the therapeutic substance and at least one viral long terminal repeat and polyadenylation signal downstream of the therapeutic sequence. Vectors derived from adenoviruses, i.e. viruses causing upper respiratory disease and also present in latent infections in primates, are also known in the art and may be used as appropriate. The ability of adenoviruses to attach to cells at low ambient temperatures is an advantage in the transplant setting which can facilitate gene transfer during cold preservation. Alternative means of targeted gene delivery comprise DNA-protein conjugates, liposomes, etc.

Cells or cell populations can be treated in accordance with the present invention in vivo or in vitro. For example, for purposes of in vivo treatments, p65RHD vectors can be inserted by direct infection of cells, tissues or organs in situ. For example, the vessels of an organ such as a kidney can be temporarily clamped off from the blood circulation, and the blood vessels perfused with a solution comprising a transmissible vector construct containing the modified RelA gene for a time sufficient for the gene to be inserted into cells of the organ; and on removal of the clamps, blood flow can then be restored to the organ and its normal functioning resumed.

In another embodiment, cell modification can be carried out ex vivo. Cell populations can be removed from the donor or patient, genetically modified by insertion of vector DNA, and then implanted into the patient or a syngeneic or allogeneic recipient. For example, an organ can be removed from a donor, subjected ex vivo to the perfusion step

described above, and the organ can be re-grafted into the donor or implanted into a different recipient of the same or different species.

Genetically modified endothelial cells may be administered by intravenous or intra-arterial injection under conventionally defined conditions. Tissue or organs comprised thereof may also be removed from a donor and grafted into a recipient by well-known surgical procedures. Prior to implantation, the treated endothelial cells or tissue or organs may be screened for genetically modified cells containing and expressing the construct. For this purpose, the vector construct can also be provided with a second nucleotide sequence encoding an expression product that confers resistance to a selectable marker substance. Suitable selectable markers for screening include the neo gene, conferring resistance to neomycin, or the neomycin analog G418.

Although any mammalian cell can be targed for insertion of the modified RelA (p65) DNA of the invention, including hematopoietic or stem cells amenable to somatic gene transfer (e.g. lymphocytes), endothelial cells are preferred cells for manipulation.

The recipient species will primarily be human, but not exclusively. Other mammals, such as non-human primates, may be suitable recipients.

The procedures and techniques to be used in employing the present invention are known in the art. Insofar as their preparation is not particularly described herein, the compounds, reagents, vectors, cell-lines, etc. to be used for carrying out the invention are known and readily available or may be obtained in conventional manner from known and readily available materials, or equivalent materials may be prepared in conventional manner from known and readily available materials.

The following Examples are illustrative only and not limitative of the invention.

Example 1

a) Subcloning of p65RHD into expression plasmid:

From plasmid pCMV4TΔp65, containing the full length cDNA of human p65 (RelA) [Ruben et al., Science 251 (1991) 1490-1493; GenBank Accession No. M62399] the p65RHD gene was derived by a PCR-based approach, using as the 5'-sequence, 5'-TAT TGG ATC CTG ACG AAC TGT TCC CCC TCA TC-3' , and as the 3'-sequence, 5'-TAC GTG TCG ACT ATT ATC CGC TGA AAG GAC TCT TCT TC-3'. The conditions under which PCR was carried out are 5 min. at 95°C; and 1 min. 94°C, 1 min. 55°C, 1 min. 72°C for 35 cycles.

The obtained PCR fragment was digested with Bam HI and XbaI to generate 5' overhangs, and cloned along with an oligomer coding for 10 amino acids of the human c-myc gene and having Hind III-compatible overhangs on the 5' end and BamHI-compatible overhangs on the 3' end, into HindIII/XbaI cut pRC.CMV (Invitrogen, San Diego, California, USA).

The resulting construct, pRC.CMV/p65RHD (abbreviated as "p65RHD"), codes for amino acids 2-320 of human p65 (RelA) preceded by a 13 residue sequence containing 10 residues from the human c-myc gene (used as a recognition sequence for the ATCC monoclonal antibody CRL 1729 [Evan et al., Molecular & Cellular Biology 5 (1985) 3610-3616]. Thus, p65RHD essentially comprises the "Rel homology domain" that allows specific subunit interactions as well as DNA binding.

Seq. Id. No. 1 and **No. 2** comprise, respectively, the nucleotide and amino acid sequence of p65RHD. **Fig. 1** comprises a schematic drawing of p65RHD.

Another construct comprising the wild-type human RelA (p65) gene was prepared by analogous methods and is referred to as pRC.CMV4TΔp65 (abbreviated as "p65WT").

b) Reporter constructs:

The porcine ECI-6 (also referred to as IκBα) reporter gene is described by de Martin et al., EMBO J. 12 (1993) 2773-2779. The porcine tissue factor (TF) reporter is described by Moll et al., J. Biol. Chem. 270 (1995) 3849-3857.

In the porcine ELAM-1 (i.e. E-selectin) reporter gene construct [Brostjan et al., Transpl.Proc. 28 (1996) 649-651], 3' to the start ATG site, a 3 bp insertion was made, creating an additional NdeI site. The promoter was cloned into the pMAMneo-luc plasmid vector (Clontech).

Primary BAEC grown in DMEM supplemented with 10% fetal calf serum (FCS), penicillin G (50 U/ml) and streptomycin (50 µg/ml) were seeded at 3 x 10^5 cells/30 mm well, and transfected 18-24 hours later with 700 ng of reporter plasmid in lipofectamine in DMEM without FCS according to manufacturer's protocol (GIBCO-BRL) for 5 hours. After addition of FCS to a final concentration of 10%, the cells were allowed to recover for 48 hours. The cells were then stimulated with 200 ng/ml lipopolysaccharide (LPS) (Sigma, St. Louis, Missouri, USA) for 7 hours, and extracts were made by freeze/thawing.

In one set of experiments, ECI-6 reporter plasmid was used, and the cells were co-transfected with pRC.CMV/p65RHD (p65RHD) and pCMV4TΔp65 (p65WT), in the respective nanogram amounts indicated in **Fig. 3**. The ECI-6 (IκBα) reporter was chosen because it is usually highly induced by p65 (RelA) and is therefore the most stringent measure for inhibitory activity by p65 RHD.

In another set of experiments, TF, ECI-6 and ELAM-1 reporters were each studied, and the cells were co-transfected with the nanogram amounts of p65RHD indicated in **Fig. 4A-C**.

All transfections were done in triplicates and repeated at least three times using two or more different plasmid preparations. Luciferase (Boehringer) and β-galactosidase (Tropix, Bedford, Massachusetts) activities were measured according to the manufacturer's directions using a 96-well luminescence reader (Bertold). Luciferase readings were normalized to β-galactosidase values or protein concentration to account for differences in transfection efficiency.

c) Results:

Induction of ECI-6 reporter activity by expression of RelA is found to be well-inhibited by the p65RHD construct, which lacks the C-terminal transactivation domain and contains a 10 amino acid epitope of the human c-Myc protein at the N-terminus. At equal nanogram amounts of p65WT and p65RHD, there is found to be almost complete inhibition of the ECI-6 reporter. This indicates that sufficient p65RHD enters the nucleus without induction of the cells by LPS. Even in the case of the ECI-6 (IκBα) reporter, which is induced more than 100-fold, virtual total inhibition is effected by expressing a 1:1 weight (molar) ratio of p65RHD to RelA (amounts of RelA and p65RHD were confirmed by Western blot analysis.)

Reporter activity induced by LPS (Fig. 3A-C) is also shown to be inhibited in a dose-dependent manner by p65RHD, reaching maximal levels of 100% at a 1:1 ratio of reporter plasmid to p65 RHD plasmid, with the exception of E-selectin, which is inhibited to the extent of about 80%.

Figs. 2 and 3 clearly show that the transcriptional induction of these genes is dependent on NFκB. Furthermore, the data demonstrate the strong inhibition of NFκB activity by p65RHD. In some cases (e.g. tissue factor), uninduced, basal reporter activity is reproducibly reduced by p65RHD expression. The basal transcriptional activity of these reporters may be due to low level NFκB activity in uninduced cells that may be a result of the transfection procedure.

In separate experiments, the transcriptional activity of a promoter lacking NFκB sites (RSV-βGal) was unaffected by p65RHD expression. Additionally, NFκB-independent, HIV Tat-mediated activation of the HIV-LTR was also found to be free from inhibition by p65RHD, whereas NFκB-dependent, RelA- or HTLV-1 Tax-induced HIV-LTR activation were completely inhibited.

Example 2:

a) Inducible tetracycline expression system:

Important for many uses of a genetic inhibitor of NFκB, such as p65RHD, is the ability to express the inhibitor only as needed. Homozygous null mutants of RelA are indicated to be lethal in utero [Beg et al., Nature 376 (1995) 167-170]. p65RHD expression, through a different mechanism, also operates to suppress transcription of NFκB-controlled genes. A system for temporal regulation of p65RHD expression is therefore highly desirable, since a transgenic animal expressing p65RHD in a regulated fashion can effectively represent a conditional RelA null mutant. Likewise, in therapeutic use, it may be desired to inhibit EC activation only on an intermittent basis.

An inducible expression system was employed to regulate p65RHD expression in vivo, in particular the binary plasmid system described by Furth et al. [PNAS USA 91 (1994) 9302-9306]. In this system p65RHD expression is driven by the tetracycline-sensitive transcriptional activator (tTA) and its expression is repressed by low levels of tetracycline. In particular, the system employs a first plasmid containing a bacterial, tetracycline-sensitive DNA binding protein fused to the HSV-VP16 transcriptional activation domain (tTA) expressed from a constitutive CMV promoter. A second plasmid contains 7 copies of the binding site for tTA, downstream of which the p65RHD gene is cloned into the vector.

When both plasmids are present in a cell, the tTA protein drives high level transcription of p65RHD. In the presence of tetracycline there is no expression of p65RHD and no inhibition of E-selectin reporter activity. In the absence of tetracycline, there is strong expression of p65RHD and maximal inhibition of the IκBα reporter activity.

b) Transgenic mice:

For the generation of transgenic mice the HindIII/XbaI-cut p65RHD was cloned into EcoRI/XbaI-cut pUHD10-3 (by filling-in the HindIII and EcoRI sites, respectively, with the Klenow fragment of E. coli polymerase I). The resulting plasmid was named pUHD 10-3/RHD.

Two separate founder strains were generated for tTA and p65 RHD. Crossing tTA mice with p65RHD mice results in double transgenic mice carrying both transgenes. These crossings were carried out under cover of tetracycline to prevent p65 RHD expression during embryogenesis. Mice carrying the tTA and p65RHD transgene, respectively, were identified by Southern blotting.

As shown in **Fig. 4**, RelA-mediated induction of the IκBα reporter is completely inhibited by p65 RHD expressed in the absence of tetracycline, whereas no inhibition is observed in the presence of tetracycline which represses expression of p65RHD.

To determine the subcellular localization of p65RHD in endothelial cells stably transfected with the p65 RHD expression plasmid under tetracycline regulation, a c-Myc specific monoclonal antibody is used. The results shown in **Fig. 5**, demonstrate that the p65 RHD protein is localized predominantly in the nucleus.

Mice that express p65 RHD in EC can be used as donors for xenotransplantation (heart and/or kidney) into rats for modelling purposes.

Example 3: Inhibition of inducible endogenous endothelial cell gene expression by regulated expression of p65RHD

To analyze the effect of NFκB inhibition on endogenous gene expression, bovine aortic endothelial cells were generated which express p65 RHD in a doxycycline-inducible manner. Doxycycline is an analog of tetracycline, which also may be used to induce expression in the system described by Gossen et al., Science 268 (1995) 1766-1769.

Transfections were done as described above using third passage BAEC. The molar ratio of plasmid pUHD172-1neo and pUHD10-3/RHD used in the transfection was 1:4. 24 hours after transfection, cells from three 30 mm wells were trypsinized, pooled, seeded into 48-well plates, and stable transfectants were selected using 600 µg/ml Geneticin (Life Technologies, Grand Island, NY, USA) for 14 days. Only wells with one colony were used for further experiments. To determine expressing clones, cells were incubated with medium containing 2 µg/ml doxycycline (Sigma, St. Louis, MO, USA) for 24 hours and stained for p65RHD as described below.

For Northern and Western blot analysis stably transfected BAEC were incubated with 2 µg/ml doxycycline for 24 hours prior to addition of LPS (200 ng/ml) for additionally 2 hours. Total RNA and protein were extracted using TRIzol (Life Technologies, Grand Island, NY, USA) according to the manufacturer's instructions. 20 µg of RNA were separated on an agarose gel containing formaldehyde, transferred to a Hybond-N nylon membrane (Amersham Life Science Inc., Arlington Heights, IL, USA) and analyzed by hybridization to radiolabeled cDNA probes of porcine IκBα, bovine E-selectin and bovine P-selectin. All membranes were probed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA to correct for unequal loading and all quantitated IκBα, E-selectin and P-selectin transcript levels were adjusted accordingly.

For Western blot analysis equal amounts of protein were boiled in SDS sample buffer and electrophoresis under denaturing conditions was carried out on a 10% polyacrylamide gel. After transfer to a PVDF (polyvinyldifluoridine) membrane (Immobilon P, Millipore, Bedford, MA, USA) by electroblotting and probing with a polyclonal antibody directed against the N-terminal region of human RelA (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) bands were visualized using horseradish

peroxidase conjugated donkey anti-rabbit IgG (Pierce, Rockford, IL, USA) and the Enhanced ChemiLuminescence assay (Amersham Life Science Inc., Arlington Heights, IL, USA) according to the manufacturer's instructions.

Results are shown in **Fig. 5**: the upper panel (A) shows a Northern blot analysis of expression of E-selectin, P-selectin and IκBα. In the absence of doxycycline these genes are well inducible by LPS, whereas their induction is strongly inhibited by doxycycline-induced expression of p65RHD. As a control, GAPDH expression was also analyzed and is not changed by either LPS or Dox treatment. The lower panel (B) shows the Western blot decorated with a N-terminal RelA-specific antibody that detects both endogenous RelA (upper band) and p65RHD. There is some detectable p65RHD expression in the absence of doxycycline which is greatly inhanced upon doxycycline treatment. The low level of p65RHD expression does not influence gene expression in these cells (lower panel).

This experiment shows that, in addition to reporter constructs, endogenous genes are also inhibited by p65RHD.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Sandoz Ltd.
(B) STREET: Lichtstrasse 35
(C) CITY: Basle
(E) COUNTRY: Switzerland
(F) POSTAL CODE (ZIP): CH-4002
(G) TELEPHONE: 61-324 5269
(H) TELEFAX: 61-322 7532
(ii) TITLE OF INVENTION: GENE THERAPY WITH MODIFIED p65 PROTEINS
(iii) NUMBER OF SEQUENCES: 2
(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)
(v) CURRENT APPLICATION DATA:
APPLICATION NUMBER: WO PCT/EP96/....
(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 60/004339
(B) FILING DATE: 26-SEP-1995

(2) INFORMATION FOR Seq. Id No. 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 999 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iii) ANTI-SENSE: NO
(v) FRAGMENT TYPE: internal
(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..999

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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            20                  25                  30

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CCA GGG ACA GTG CGC ATC TCC CTG GTC ACC AAG GAC CCT CCT CAC CGG         288
Pro Gly Thr Val Arg Ile Ser Leu Val Thr Lys Asp Pro Pro His Arg
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CCT CAC CCC CAC GAG CTT GTA GGA AAG GAC TGC CGG GAT GGC TTC TAT         336
Pro His Pro His Glu Leu Val Gly Lys Asp Cys Arg Asp Gly Phe Tyr
            100                 105                 110

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145                 150                 155                 160

CGT GGG GAC TAC GAC CTG AAT GCT GTG CGG CTC TGC TTC CAG GTG ACA        528
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        195                 200                 205

TGC CGA GTG AAC CGA AAC TCT GGC AGC TGC CTC GGT GGG GAT GAG ATC        672
Cys Arg Val Asn Arg Asn Ser Gly Ser Cys Leu Gly Gly Asp Glu Ile
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225                 230                 235                 240

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Thr Gly Pro Gly Trp Glu Ala Arg Gly Ser Phe Ser Gln Ala Asp Val
                245                 250                 255

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His Arg Gln Val Ala Ile Val Phe Arg Thr Pro Pro Tyr Ala Asp Pro
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    290                 295                 300

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(2) INFORMATION FOR Seq. Id. No. 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 332 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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            20                  25                  30
Val Glu Ile Ile Glu Gln Pro Lys Gln Arg Gly Met Arg Phe Arg Tyr
        35                  40                  45
Lys Cys Glu Gly Arg Ser Ala Gly Ser Ile Pro Gly Glu Arg Ser Thr
    50                  55                  60
Asp Thr Thr Lys Thr His Pro Thr Ile Lys Ile Asn Gly Tyr Thr Gly
65                  70                  75                  80
Pro Gly Thr Val Arg Ile Ser Leu Val Thr Lys Asp Pro Pro His Arg
                85                  90                  95
Pro His Pro His Glu Leu Val Gly Lys Asp Cys Arg Asp Gly Phe Tyr
            100                 105                 110
Glu Ala Glu Leu Cys Pro Asp Arg Cys Ile His Ser Phe Gln Asn Leu
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145                 150                 155                 160
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His Pro Ile Phe Asp Asn Arg Ala Pro Asn Thr Ala Glu Leu Lys Ile
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    290                 295                 300
Asp Asp Arg His Arg Ile Glu Glu Lys Arg Lys Arg Thr Tyr Glu Thr
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Phe Lys Ser Ile Met Lys Lys Ser Pro Phe Ser Gly
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```

Claims:

1. A modified human p65 protein transdominantly inhibiting the wild-type p65 protein, or a corresponding DNA coding therefor.

2. A protein according to claim 1 having the transcription activation domain of the wild-type protein substantially dysfunctional or deleted, or corresponding DNA coding therefor, optionally with ancillary sequences for quantification or recognition.

3. A protein or corresponding DNA coding therefor, comprising or coding for, essentially, amino acids 1-320 or 2-320 of the wild-type human p65 amino acid sequence.

4. The protein according to claim 3 having the amino acid sequence of Seq. Id. No. 2, or the DNA coding therefor having the nucleotide sequence of Seq. Id. No. 1, or the sequence obtained by adding or replacing one to several amino acids in Seq. Id. No. 2.

5. A vector construct for achieving delivery of a modified protein according to claim 1 or 3, or of corresponding DNA coding therefor, to appropriate recipient cells, tissue or organs.

6. A vector according to claim 5 comprising a regulable element in the form of a tetracycline-inducible promoter.

7. A method of genetically modifying mammalian cells to render them less susceptible to an inflammatory or immunological stimulus, which comprises inserting therein, or the progenitors thereof, DNA encoding a transdominant inhibitor of a RelA (p65) protein, whereby transcriptional activation of inflammatory genes is suppressed under endothelial cellular activating conditions.

8. A method of transplanting donor allogeneic or xenogeneic mammalian cells, or tissue or organs to a mammalian recipient in whom such cells, tissue or organs are subject to inflammatory or immune activation, which comprises:

(a) genetically modifing the donor cells or progenitor cells thereof or tissue or organ by inserting therein DNA encoding a transdominant inhibitor of RelA(p65) protein;

(b) implanting the resultant modified donor cells, tissue or organ into the recipient; and

(c) expressing in the resultant modified cells, tissue or organ, functionally active transdominant inhibitor, whereby transcriptional activation of inflammatory genes therein is suppressed.

9. Graftable mammalian endothelial cells, tissue or organs comprising DNA encoding a transdominant inhibitor of the endogenous RelA (p65) protein.

10. A non-human transgenic mammal comprising DNA encoding a transdominant inhibitor of a RelA (p65) protein.

Figure 1:

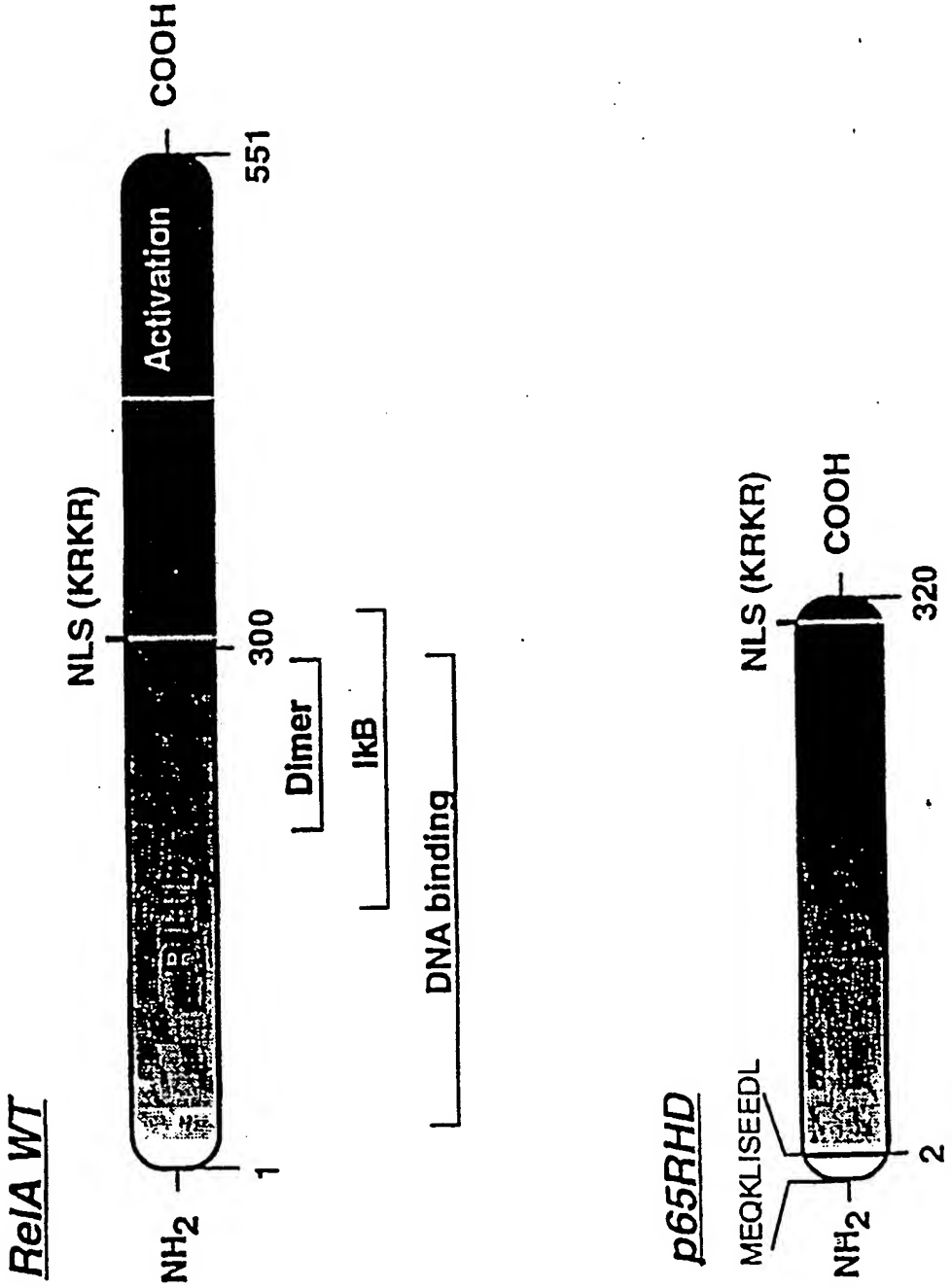

RelA WT
NH2
1
NLS (KRKR)
300
Activation
551
COOH
Dimer
IkB
DNA binding
p65RHD
MEQKLISEEDL
NH2
2
NLS (KRKR)
320
COOH

Figure 2:

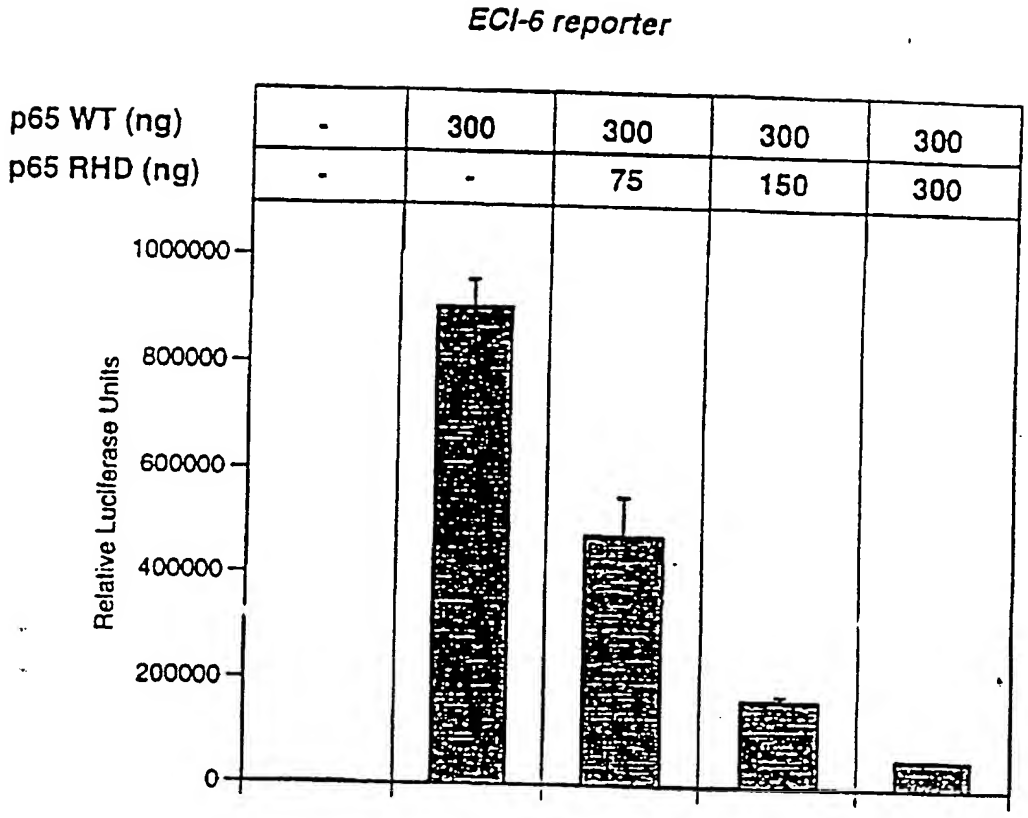
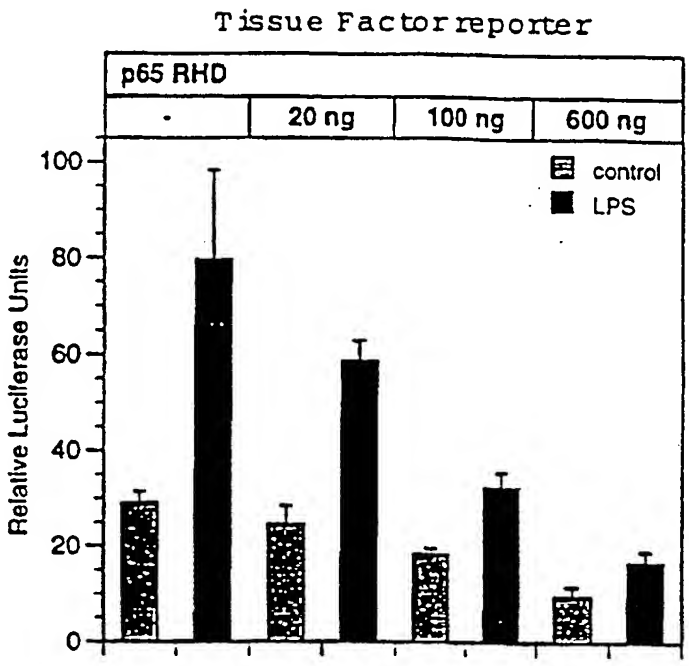

ECI-6 reporter
p65 WT (ng)
p65 RHD (ng)
-
300
300
300
300
-
-
75
150
300
Relative Luciferase Units
1000000
800000
600000
400000
200000
0

Figure 3 (sheet 1/2):

A



B

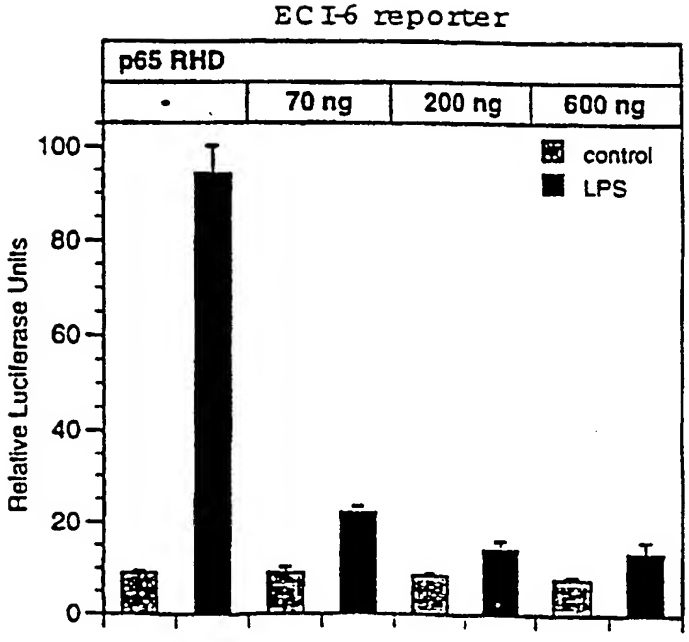


Figure 3 (sheet 2/2):

C

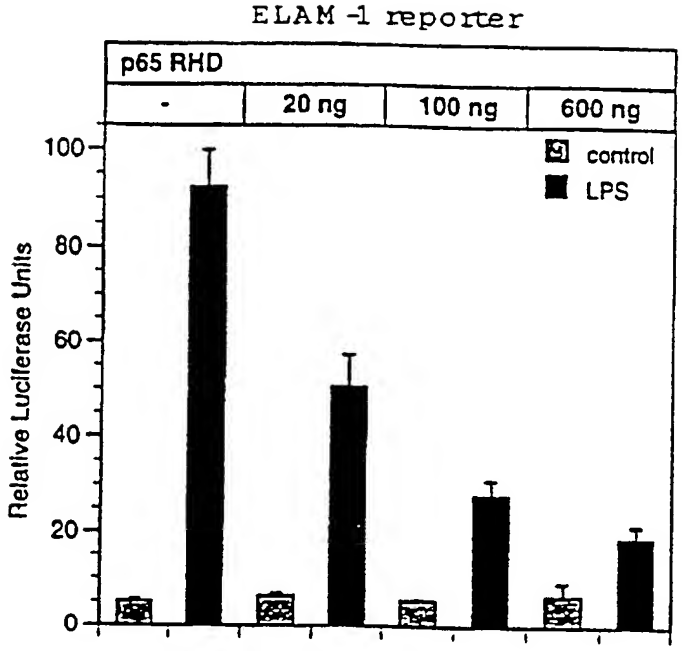


Figure 4:

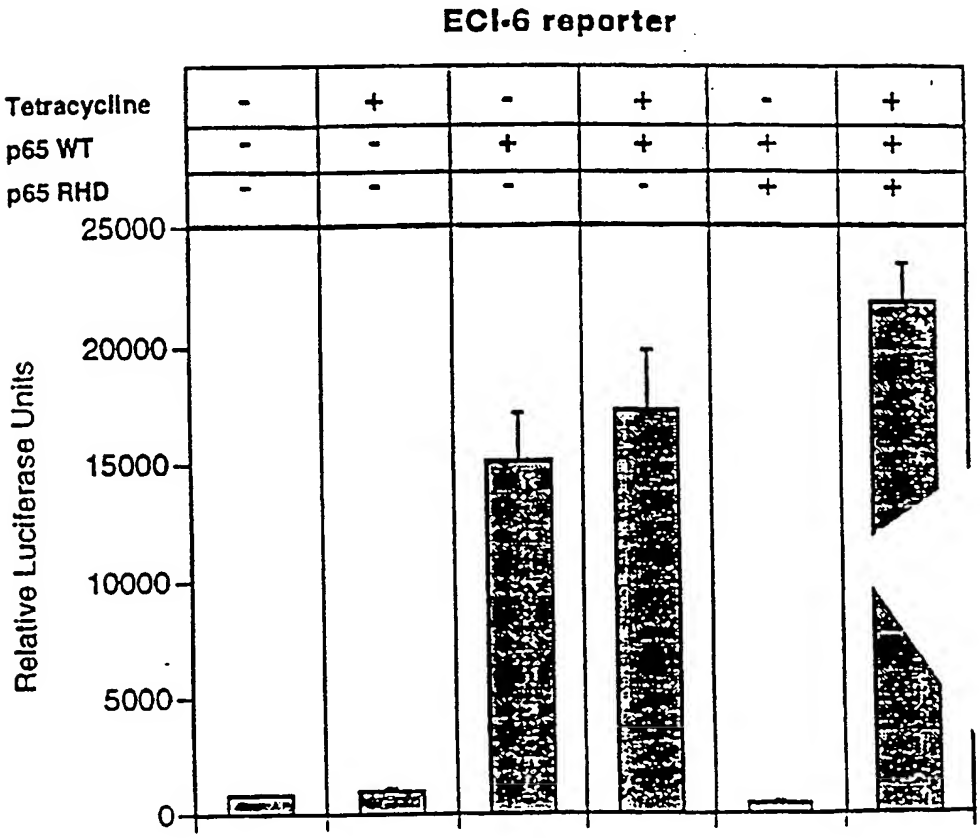
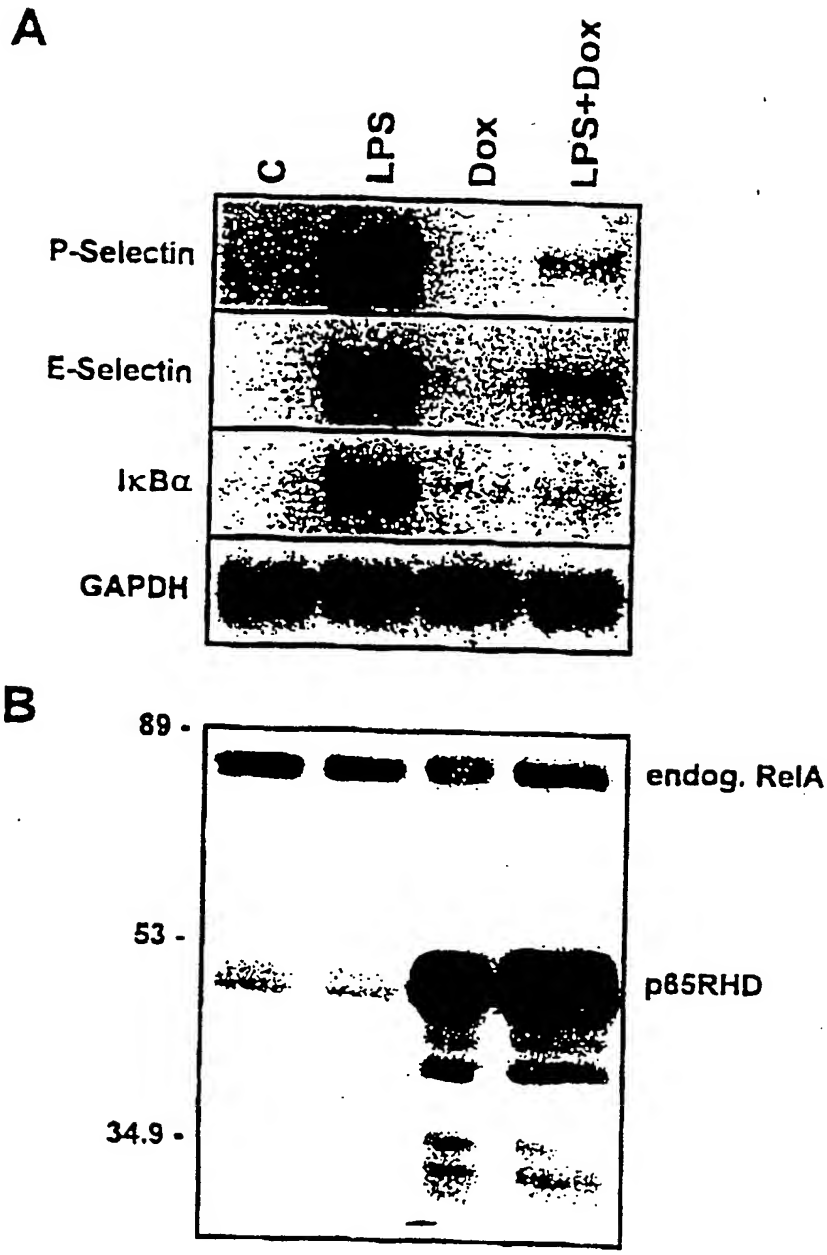

ECI-6 reporter
Tetracycline
p65 WT
p65 RHD
- + - + - +
- - + + + +
- - - - + +
Relative Luciferase Units
25000
20000
15000
10000
5000
0

Figure 5:


A
C
LPS
Dox
LPS+Dox
P-Selectin
E-Selectin
IκBα
GAPDH
B
89 -
53 -
34.9 -
endog. RelA
p65RHD

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/EP 96/04216

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 A01K67/027 C12N5/10 A61K48/00 C07K14/47

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SCIENCE, vol. 259, 26 March 1993, LANCASTER, PA US, pages 1912-1915, XP000616266 SUN, S.C. ET AL.: "NF-kappaB controls expression of inhibitor I kappa B alpha: evidence for an inducible autoregulatory pathway" see page 1914, paragraph 2 - page 1915, column 1 ---	1-5
X	WO,A,93 20219 (US HEALTH) 14 October 1993 see page 4, line 14 - line 16; claims 1,2,10-13,22; figure 4C see page 15, line 25 - line 33 see page 10, line 11 - page 12, line 9 --- -/--	1

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 January 1997	03.02.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Chambonnet, F

Form PCT/ISA/210 (second sheet) (July 1992)

Int. ional Application No
PCT/EP 96/04216

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MOLECULAR AND CELLULAR BIOLOGY, vol. 13, no. 2, February 1993, pages 852-860, XP000614400 TOLEDANO, M.B. ET AL.: "N-terminal DNA-binding domains contribute to differential DNA-binding specificities of NF-kappaB p50 and p65" see the whole document	1

INTERNATIONAL SEARCH REPORT

I· -national application No.
PCT/EP 96/04216

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 8 and, partially, 7
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although these claims are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 96/04216

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9320219	14-10-93	AU-A- 4045193	08-11-93